ARMY MEDICAL RESEARCH LABORATORY

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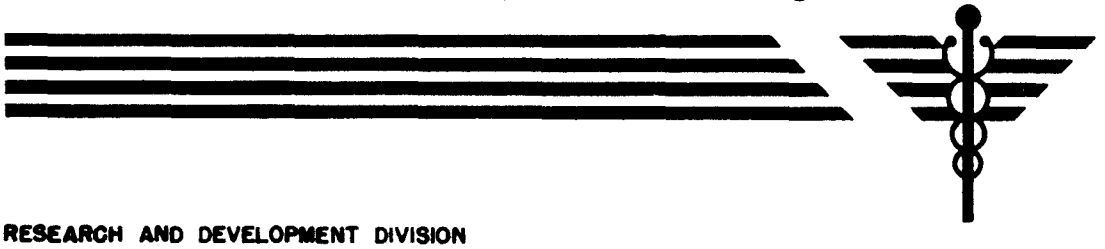
THE USE OF X-RAY INDUCED CYTOLOGICAL CHANGES IN ALLIUM ROOT TIP CELLS AS INDICES OF THE EFFECTS OF VARIOUS PROTECTIVE SUBSTANCES*

*Subtask under Biological Aspects of Ionizing Radiation, AMRL Report No. 6-59-08-014, Subtask, Early Effects of Ionizing Radiation.



RESEARCH AND DEVELOPMENT DIVISION
OFFICE OF THE SURGEON GENERAL
DEPARTMENT OF THE ARMY

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by

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from

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ABSTRACT

THE USE OF X-RAY INDUCED CYTOLOGICAL CHANGES IN ALLIUM ROOT TIP CELLS AS INDICES OF THE EFFECTS OF VARIOUS PROTECTIVE SUBSTANCES

OBJECT

To determine the correlation between the postirradiation selection times, the mitotic index and frequency of chromosome abnormalities in Allium cepa root tip cells for possible use as a means of measuring the influence of various substances on radiation damage under controlled conditions.

RESULTS AND CONCLUSIONS

The time and degree of mitotic inhibition showed that the mitotic index reached a minimum at about four to six hours after irradiation with x-ray doses of 50 to 800 roentgens. The highest per cent of chromosomal abnormalities for the same dose range was found at four hours after irradiation. At 36 hours after irradiation, the dose-effect curve for chromosomal abnormalities became approximately linear. At room temperature (20-23°C) the mitotic time for Allium cepa root tip cells was approximately four hours and the intermitotic time was 43 hours. Preliminary experiments with cysteine and glutathione showed a protective effect against the radiation induced damage, indicating that these cytological changes can be used as indices of the effects of various protective substances.

RECOMMENDATIONS

Detailed investigations can be made on the influence of various chemicals on the cytological changes induced by different ionizing radiations using mitotic inhibition and the frequency of chromosome abnormalities in Allium cepa as criteria of radiation damage.

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I. INTRODUCTION

An important objective in the applied phase of radiobiology is the protection of biological material from the damaging effects of ionizing radiations. Methods of evaluating the protective effects of various substances are continuously being sought. There have been many methods developed which can be used to assess the value of agents in protecting against the cytological changes induced by ionizing radiations. Many of these, however, require elaborate equipment and are very time-consuming. Some of these measures are: 1) mitotic inhibition - Carlson (1), Friedenwald, et al. (2), Knowlton & Hempelmann (3), Marshak (4), Marshak and Bradley (5), Rasch (6), and Luther & Krebs (7); 2) the frequency of chromosomal aberrations - Sax (8, 9, 10), Sax and Brumfield (11), and Sparrow and Rubin (12); 3) nucleic acid content - Sparrow, Moses and Dubow (13); 4) bioelectric potential - Bless (14); and 5) growth rate - Quastler, et al. (15). These reports indicate that a measurement of mitotic inhibition and the frequency of chromosomal abnormalities might be most accurate and efficient criteria for evaluating the influence of various chemical agents on radiation damage. However, before these measurements can be applied, certain criteria of radiation damage must be established.

Mitotic abnormalities can be induced by a great number of conditions. These can be grouped as follows: 1) ionizing radiations (1, 2, 3, 4, 5, 6), 2) non-ionizing radiations (16); 3) ultrasonics (17), 4) chemicals (18, 19, 20); and 5) age and genetic factors (21). In the present study the effects of ionizing radiation on root tip cells of Allium cepa were systematically investigated.

II. EXPERIMENTAL

A. Apparatus and Methods

The Allium cepa root tips were germinated from bulbs suspended in a container of tap water through which air was continuously bubbled. The roots generally reached a length of 2 to 5 cm prior to irradiation. All experiments were carried out at temperatures ranging between 20 and 23°C.

The x-ray unit used was that of the Kelley-Koett Manufacturing Company and was operated at 200 KV and 6 ma. The target distance

was 30cm, and the x-ray intensity as measured by a Victoreen dosimeter was 40 roentgens per minute. Filters used were 1 mm Al and 1/2 mm Cu. Dosage was increased by lengthening the time of exposure.

A consecutive series of x-ray doses were used. These were 50, 100, 200, 400, 600 and 800 roentgens, and were obtained by exposing the material to the x-rays for 1.25, 2.5, 5.0, 10.0, 15.0 and 20.0 minutes respectively. Three bulbs were used for each dosage level. The entire root systems including the bulbs were subjected to the x-rays. Twelve unirradiated control bulbs were studied simultaneously. The bulbs to be irradiated were wrapped in wet paper toweling and placed in a lucite container to prevent dessication during irradiation. One root was selected from each bulb and fixed in a 3:1 solution of absolute alcohol and acetic acid at the desired time after irradiation. These times were 2, 3, 4, 6, 8, 12, 16, 24, and 36 hours post-irradiation. After staining each root, using the Feulgen technique, a small piece of meristematic tissue, usually about 1 mm length, just back of the root cap was removed and squashed on a slide. Neither cells of the root cap nor cells in the regions of elongation and differentiation were involved in these studies.

The effects of ionizing radiations were measured in two ways. The first involved the effect of irradiation on the mitotic index. The mitotic index was determined by classifying the first 500 cells encountered on each slide as either mitotic or non-mitotic. The values were obtained by pooling the data of the three roots for each time period and expressing them in per cent of the total number of cells. A total number of 20,500 cells was used for the normal mitotic index value and 1500 cells for each point on the inhibition curves. The other method involved abnormalities in the chromosomes themselves. Since these abnormalities were most evident in late anaphase and early telophase, only these two phases of mitosis were considered. In making the counts, anaphase and telophase nuclei were classified either as those showing one of the three general types of aberration (fragments, bridges, or both fragments and bridges) or those showing normal division figures. The values presented in this report are from pooled data, and are given in percentage values with the standard errors. The total number of cells counted for any given pooled value ranged between 30 and 443, depending on the time of selection and the x-ray doseage.

In a preliminary experiment on the protective effects of some chemicals, some bulbs were placed in 0.0001 M solutions of the chemicals (cysteine and glutathione) for 24 hours prior to irradiation.

B. Results

Table 1 and Figure 1 show the degree and the time of mitotic inhibition depending on the applied x-ray dose. Three events may be emphasized: 1) a fall in the mitotic index (to zero or nearly zero at the higher levels of radiation); 2) a rise in the count to a peak, usually to about the same level or higher than the control values; and 3) a secondary fall in the count from which a slow recovery was indicated for the lowest levels of radiation. The primary fall was probably due to the inhibition of the first mitosis after irradiation, and the secondary fall was the result of the death of many of the cells which undertook the first mitosis (Luther and Krebs (7)).

TABLE 1

EFFECT OF VARIOUS DOSES OF X RAYS ON THE MITOTIC INDEX OF ALLIUM CEPA ROOT TIP CELLS AT VARIOUS TIMES AFTER IRRADIATION (Values in per cent with standard error, based on 1500 cells per point)

Hours after Irradiation	X-RAY DOSE									
	50 r		100 r		200 r		600 r		800 r	
2	10.5	0.8	8.3	0.7	4.8	0.6	5.2	0.6	3.9	0.5
3	7.1	0.7	6.6	0.6	2.4	0.4	2.3	0.4	2.1	0.4
4	7.1	0.7	5.8	0.6	1.9	0.4	1.6	0.3	1.2	0.3
6	8.7	0.7	3.7	0.5	1.4	0.3	1.3	0.3	0.1	0.1
8	9.5	0.8	13.7	0.9	2.4	0.4	0.2	0.1	0.0	0.0
12	12.1	0.8	11.4	0.8	5.9	0.6	0.2	0.1	0.0	0.0
16	7.9	0.7	8.6	0.7	9.7	0.8	0.9	0.2	0.0	0.0
24	9.1	0.7	6.3	0.6	5.7	0.6	11.8	0.8	0.1	0.1
36	10.6	0.8	6.4	0.6	6.5	0.6	9.2	0.7	6.7	0.6

The primary fall of the mitotic index can be used for the determination of the mitotic time (23). For this purpose the slopes of the inhibition occurring with the higher doses, determined by the method of least squares, were extrapolated to their intersections with the x-axis. Figure 2 shows that the ends of the extrapolated slopes of mitotic decline fell around four hours for the onion root tip cells. This indicates that the last cell to pass the critical point at the time of irradiation would have completed division four hours later. Since Allium cepa is most sensitive at the onset of prophase (Marshak, 4) this is believed to establish the mitotic time at four hours.

Table 2 and Figure 3 present the frequency of chromosome abnormalities for different doses at various times after irradiation. For every dose studied the highest percentage of observed abnormalities was at four hours after irradiation. The frequency of spontaneous chromosomal abnormalities (counts made from fifty unirradiated root tips) was found to be less than 0.01%, and consequently not significant.

Figure 4 presents the dose effect correlation for the time intervals of 4 and 36 hours after irradiation. It shows that for the "structural" effect proportionality between dose and effect exists at 36 hours after irradiation

The bulbs treated with 0.0001 M solutions of cysteine and glutathione showed a definite change in radiosensitivity of the root tip cells. In the cysteine treated tissue the frequency of chromosomal aberrations produced by 200 r was, for example, about 44% lower than in the water grown controls and glutathione treated onion roots showed a 21% protection when compared to the controls.

TABLE 2

EFFECT OF VARIOUS DOSES OF X-RAYS ON THE FREQUENCY OF CHROMOSOME ABNORMALITIES IN ALLIUM CEPA ROOT TIP CELLS AT VARIOUS TIMES AFTER IRRADIATION. (Values in per cent with standard error, based on 30 - 443 cells per point.)

Hours after irradiation.	X-RAY DOSE					
	50 r	100 r	200 r	400 r	600 r	800 r
2	14.6±1.8	37.6±3.3	55.4±5.8	--	--	--
3	16.2 2.7	52.6 3.2	80.9 5.0	81.8±6.7	83.3±6.8	83.3±6.8
4	20.8 4.8	71.2 5.3	86.0 3.5	81.1 5.4	92.8 3.5	97.1 2.8
6	17.5 2.5	35.2 5.7	--	--	--	--
8	13.6 1.7	12.9 1.6	--	--	--	--
12	9.1 1.6	34.3 3.0	67.3 5.1	--	--	--
16	6.9 1.5	21.5 2.6	--	--	--	--
24	5.1 1.1	17.9 2.6	54.5 5.7	--	--	--
36	6.3 1.7	12.1 1.6	28.3 3.6	47.1 4.3	79.8 2.1	92.4 2.1

III. DISCUSSION

Allium cepa was selected as a test agent for the following reasons. The nuclei are relatively large, being about 15 microns in diameter and the chromosomes are large and relatively few in number, 16 in the diploid condition. Allium cepa is easily obtained throughout the

year. The bulbs remain viable for long periods of time and the roots are easily produced. The stained root is handled with ease and the embryonic root tip may be separated from the region of elongation without difficulty.

X-rays influence the mitotic process, in general, in the following manner. The first apparent response to x-rays is a temporary inhibition of nuclear division as shown for Chortophaga by Carlson (1) and for Tradescantia by Sax (8) and Sparrow and Rubin (12). This inhibition has been found to be proportional to the x-ray dose. There is a period of increased mitotic activity following the inhibition which is also proportional to the dose for chick tissue culture (Canti and Spear (22)), rat cornea (Friedenwald (2)), and Chortophaga neuroblasts (Carlson (1)).

The cells of Allium cepa respond to x-irradiation in a manner similar to that found for the above mentioned cells. Doses of 50,100, 200, 600 and 800 roentgens, produce in root tips, at various time intervals after irradiation, the characteristic correlation between dose and mitotic inhibition (See table 1 and figure 1). There also was some increase in mitotic activity following the period of inhibition. The mitotic time could be determined from the fall in mitotic activity. If the mitotic index is known the intermitotic time can be calculated, by using the formula:

$$\text{Intermitotic Time} = \frac{\text{Mitotic Time}}{\text{Mitotic Index}}$$

The mitotic index is obtained by determining the average number of cells in division at any one time in normal tissue. Counts made on over 20,000 onion root tip cells gave for this index the value of 9.2% With this figure and the value of 4 hours for the mitotic time the intermitotic time was determined to be 43 hours. The total length of the mitotic cycle would thus be about 47 hours. A per cent breakdown of the counted mitotic phases have the values: prophase - 37.7%; metaphase - 27.4%; anaphase - 9.9%; and telophase - 25%.

Converting these per cent values to time in minutes, the duration for the mitotic phases of the root tip cells are in minutes: prophase-90; metaphase - 66; anaphase - 24; and telophase - 60. Marshak (4) has stated that the time interval from onset of prophase to late anaphase is 3.0 hours, with which the reported findings agree. The length of the intermitotic time is also in agreement with that reported by Marshak. The length of the intermitotic time is also in agreement with the findings of Woods (24) who used a different method involving the use of an anti-metabolite (5-amino-uracil), a mitotic inhibitor.

In establishing dose effect correlations with respect to the frequency of chromosome abnormalities (Figure 3) there must be a distinction

made between the "physiological" effect and the "structural" effect. The "physiological" effect is produced in cells in division during irradiation and is characterized by clumping and fusion of chromosomes (Sax, 9). The "structural" effect is produced in cells that are in the inter-phase stage at the time of irradiation. It involves chromosome aberrations resulting from single breaks and/or two breaks. Single break aberrations include single deletions and are detected by the presence of paired fragments at anaphase or by a single bridge and accompanying fragment. Chromosome aberrations resulting from two breaks are indicated by the presence of dicentric chromosomes at metaphase, dicentric bridges at anaphase, or ring chromosomes with their accompanying fragments. Translocations and inversions are difficult to detect in Allium (Sax (9), Rasch (6)). In relatively simple systems like Tradescantia microspores, the aberrations involving two breaks (rings or dicentrics) vary in frequency with the square of the dose when the time of exposure is kept constant, but increase with the 1.5 power of the dose if the radiation intensity is held constant (Sax, (9)). Since the determination of dose effect curves for aberrations induced in root tip chromosomes is generally more difficult due to greater variability and to difficulties in classifying the different types, Sax restricted his studies to the observation of rings and dicentrics. In his work with Allium Sax used 3 doses (150, 300, 600r) and found the frequency of aberrations among 815 anaphases 4 days after irradiation to be: 4% for 150 r; 9% for 300 r, and 27% for 600 r. Although these dose effects are not as consistent as those determined for Tradescantia microspore chromosomes, it is apparent to Sax that the aberration frequency increased approximately as the 1.5 power of the dose. Rasch, using 100, 1000 and 10,000 r and observing the cells 2, 3, and 4 days after irradiation found dicentric and ring aberrations per cell to be 0.11 for 100 r, 0.27 for 1000 r, and 1.69 for 10,000 r and dicentric and ring fragment aberrations per cell to be 0.41 for 100 r; 0.74 for 1000 r; and 5.67 for 10,000 r. From these values, using Newcomb's formula, Rasch concluded that the aberration frequency increased approximately as the 0.6 power of the dose.

The detailed findings presented in this study (linearity between dose and effect at 36 hours (figure 4)) confirm neither the value of Sax nor the value given by Rasch. This may be explained by the fact that figures 3 and 4 do not give chromosomal aberrations in the sense as defined by Sax, Catchside, Giles and others, but rather chromosomal abnormalities. Sax and the others restricted themselves in studying the dose effect correlation to certain types of abnormalities to obtain an indication of the mechanisms and the laws by which chromosomal aberrations are produced by ionizing radiation. The goal of this investigation was (restricting the studies to anaphase and telophase

nuclei with either fragments or bridges, or fragments and bridges) to obtain an evaluation preparation for the study of the effects of protective substances on the mitotic process.

IV. CONCLUSIONS

The mitotic and intermitotic times for Allium cepa root tip cells were established for the specified conditions - the mitotic time being about four hours, the intermitotic time 43 hours and the mitotic cycle 47 hours.

For mitotic inhibition studies the most suitable time for observation would be about six hours after irradiation. The degree of mitotic inhibition and the frequency of chromosomal abnormalities can be used as effective measures of the influence of different substances on radiation damage under controlled conditions. Preliminary experiments with pretreated cells (0.0001 M solutions of cysteine and of glutathione) showed a beneficial influence on the radiation induced cytological changes. The selected time for study of the frequency of structural chromosomal abnormalities should be well out of the range of the mitotic time but within the first mitotic cycle after irradiation. For Allium cepa root tip cells, a proper time is about 36 hours after irradiation.

V. RECOMMENDATIONS

Studies can be made of the influence of various chemicals on the cytological effects of ionizing radiations using mitotic inhibition and the frequency of chromosome abnormalities in Allium cepa as criteria of radiation damage.

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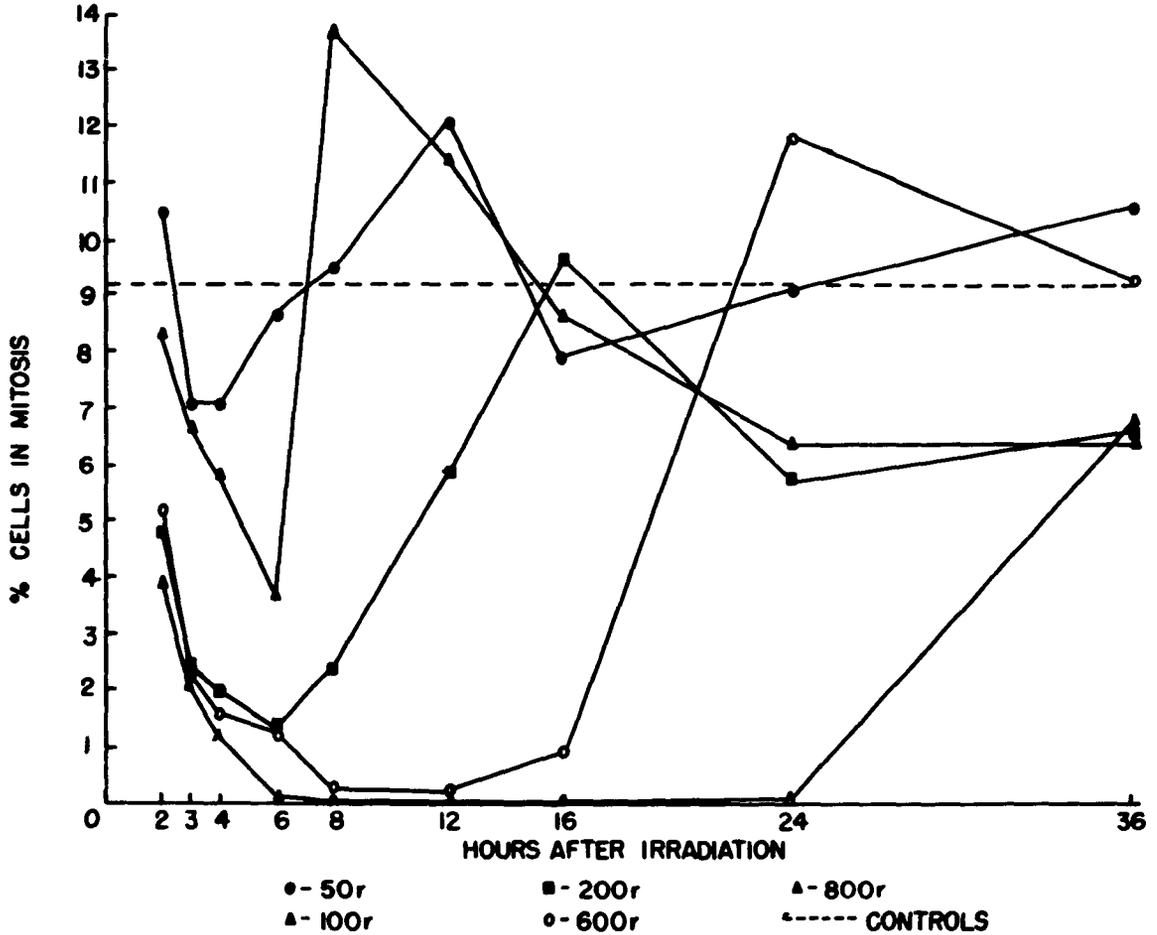

FIGURE I. THE EFFECT OF VARIOUS X-RAY DOSES ON THE MITOTIC INDEX OF *Allium Cepa* ROOT TIP CELLS AT VARIOUS TIMES AFTER IRRADIATION.

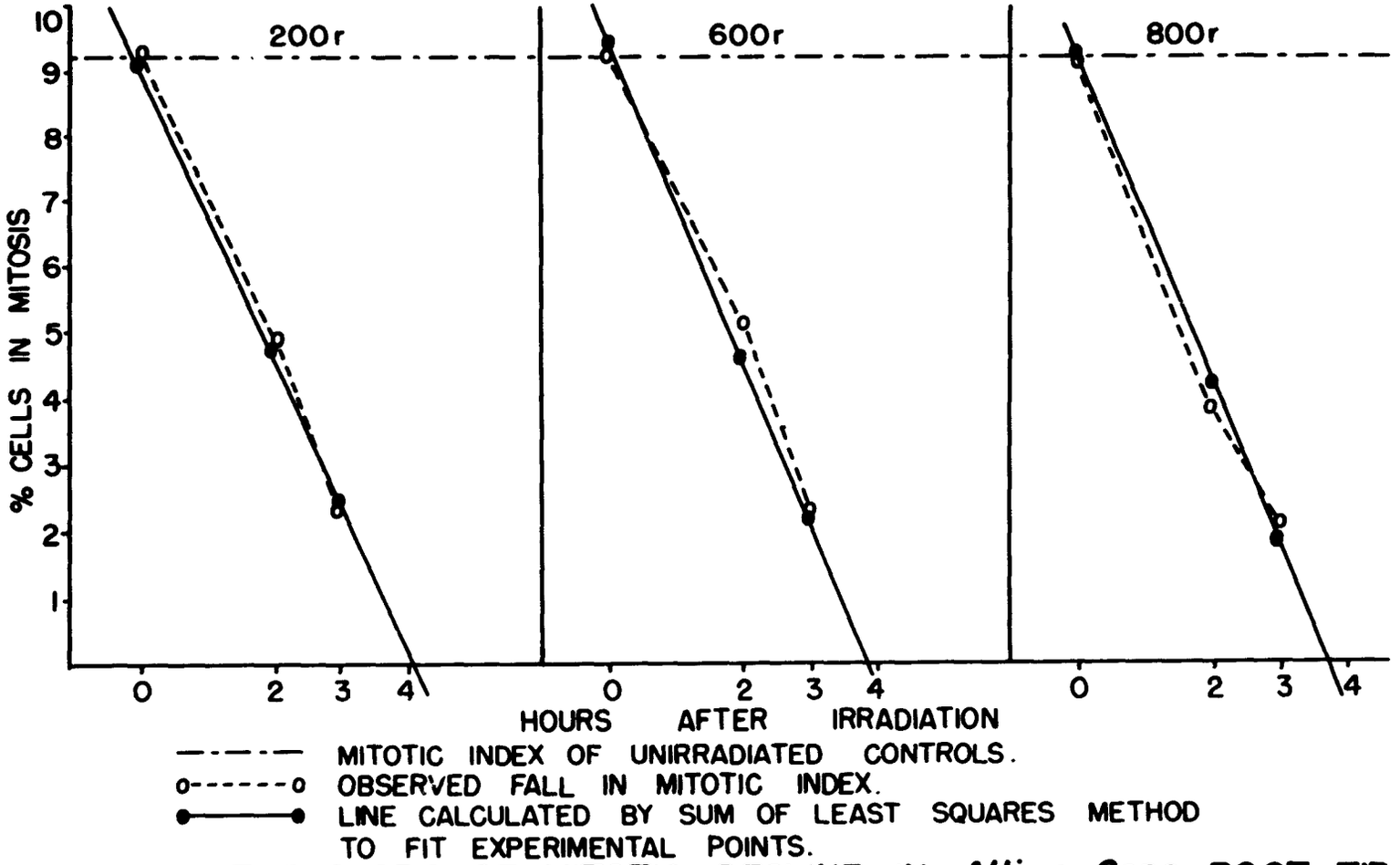

FIGURE 2. THE SLOPE OF MITOTIC DECLINE IN *Allium Cepa* ROOT TIP CELLS AFTER EXPOSURE TO VARIOUS X-RAY DOSES.

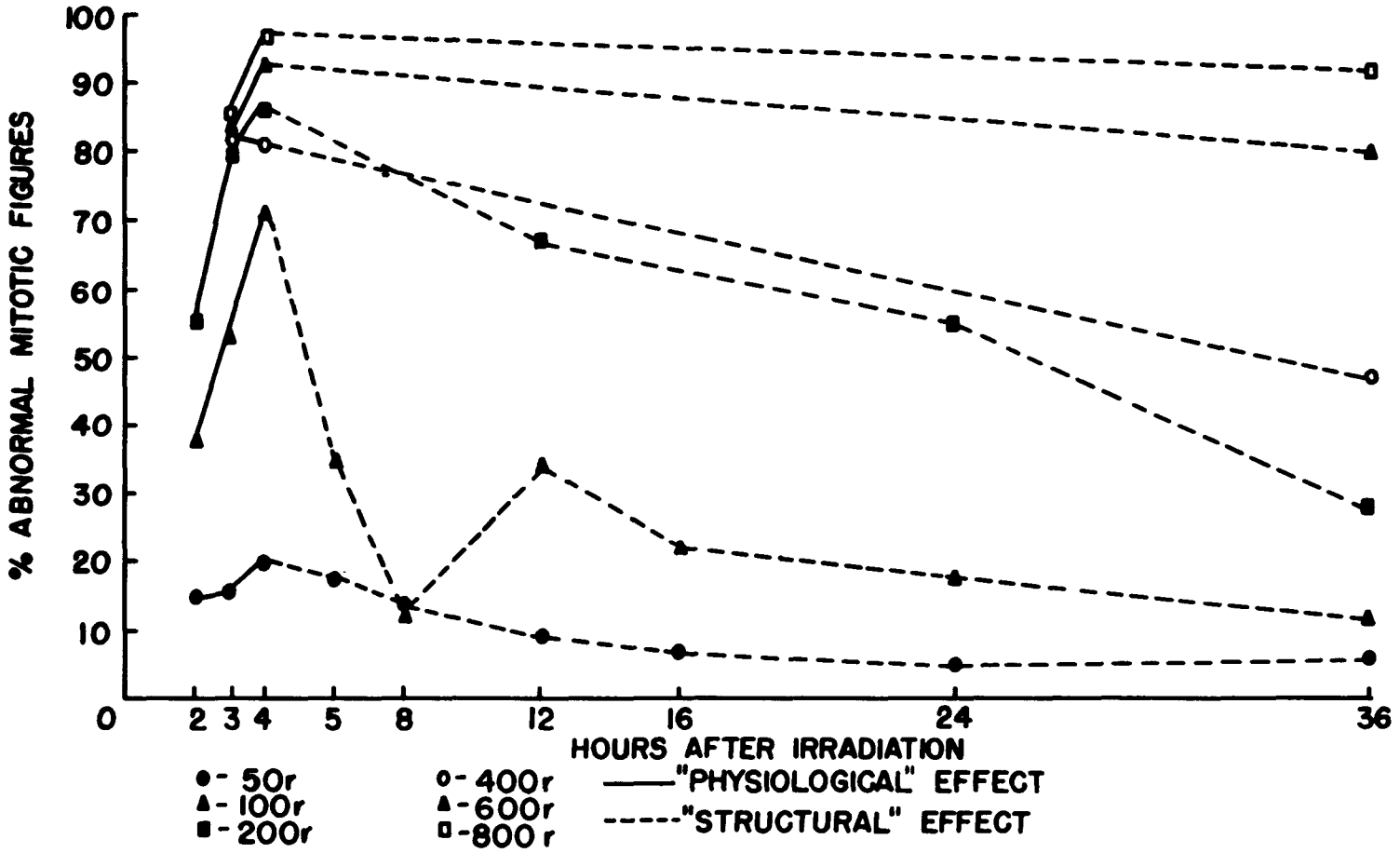

FIGURE 3. THE FREQUENCY OF CHROMOSOMAL ABNORMALITIES FOR SEVERAL X-RAY DOSES AT VARIOUS TIMES AFTER IRRADIATION IN *Allium Cepa* ROOT TIP CELLS.

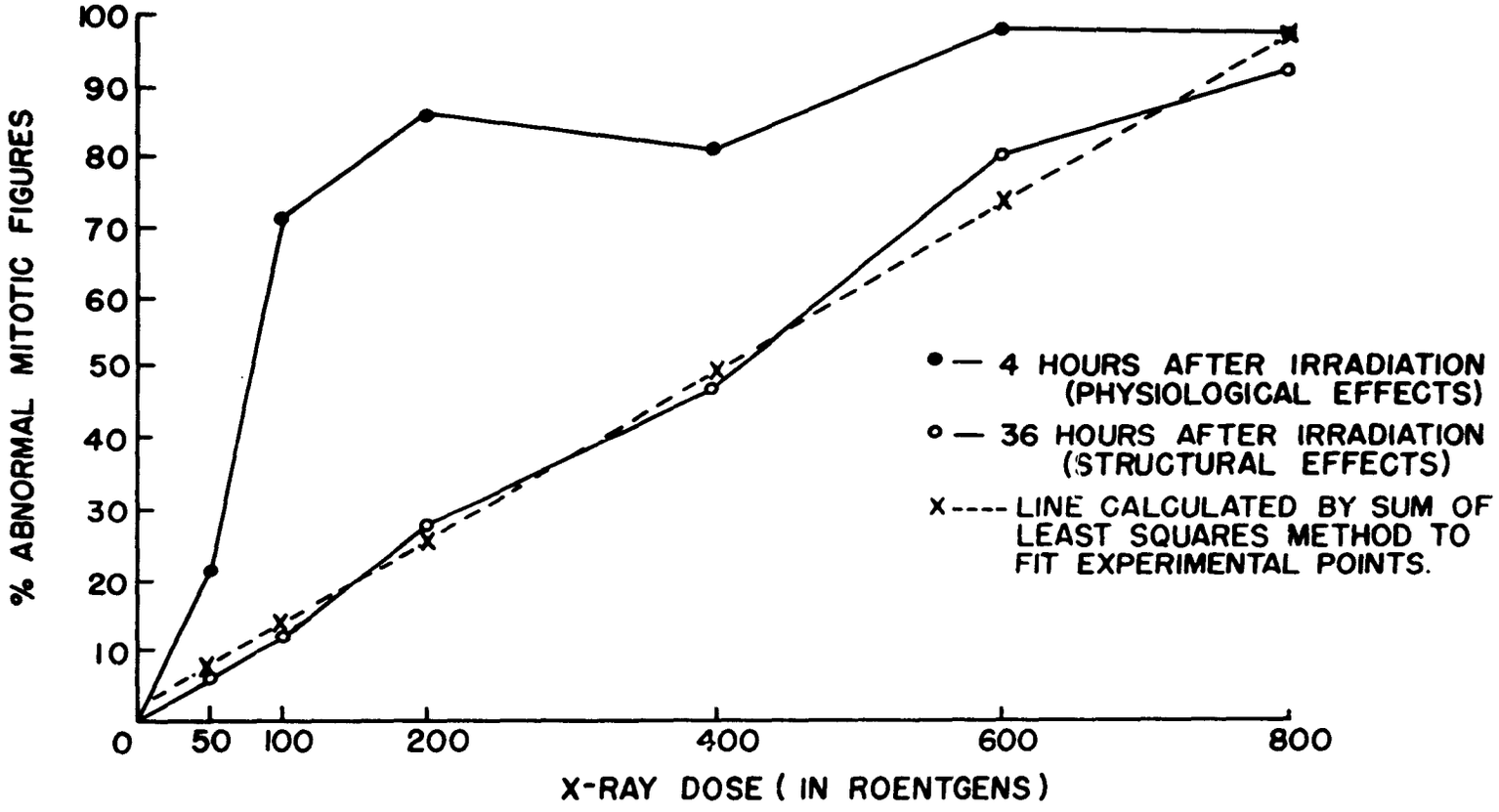

FIGURE 4. THE EFFECT OF VARIOUS X-RAY DOSES ON THE FREQUENCY OF CHROMOSOME ABNORMALITIES AT 4 AND 36 HOURS AFTER IRRADIATION.